Article

Organotypic Co-Cultures as a Novel 3D Model for Head and Neck Squamous Cell Carcinoma

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Supplementary Materials

Table S1. Currently available preclinical models for HNSCC.

Tumour / Derivation	Non-HPV induced HNSCC	HPV-induced HNSCC
Cell culture	**More than 370 HNSCC cell lines**, reviewed by Lin et al, 2007[48]	Steenbergen et al, 1995 Ferris et al, 2005 White et al, 2007 Hoffmann et al, 2008 Sartor et al, 2011 Tang et al, 2012 Forslund et al, 2019
Animal model	Peng et al, 2013[53] Kimple et al, 2013[54] Klinghammer et al, 2014[19] Li et al, 2016[55] Morton et al, 2016[56] Facompre et al, 2017[18] Karamboulas et al, 2018[57] Ruicci et al, 2019[58]	Kimple et al, 2013 Klinghammer et al, 2014 Facompre et al, 2017

Human, "personalized patient-derived"	Gerlach et al, 2014[40] Tanaka et al, 2019[12] Driehuis et al, 2019[59] Karakasheva et al, 2020[60] Engelmann et al, 2020	Tanaka et al, 2019 Engelmann et al, 2020

Abbreviations: HNSCC, Head and neck squamous cell carcinoma; HPV, human papillomavirus.

Table S2. Primary and secondary antibodies for IHC and IF. Abbreviations: IF, immunofluorescence; IHC, immunohistochemistry; cc-3, cleaved caspase-3; PanCK, pan-cytokeratin.

Method	Primary antibody	Ref#	Manufacturer	Dilution	Secondary antibody	Ref#	Manufacturer	Dilution
IHC	Anti-CD45	M0703	Dako, Denmark	1:100	HRP anti-	MP7402	Vector Laboratories, USA	ready to use
IHC	Anti-cleaved caspase-3	9661	Cellsignaling, USA	1:300	Biotinylated anti-rabbit	BA-1000	Vector Laboratories, USA	1:200
IHC	Anti-ki-67	M7420	Dako, Denmark	1:50	HRP anti-mouse	MP7402	Vector Laboratories, USA	ready to use
IHC	Anti-PanCK	GP14	Progen, Germany	1:200	Biotinylated anti-guinea-pig	BA-7000	Vector Laboratories, USA	1:200
IF	Anti-PanCK	GP14	Progen, Germany	1:50	Alexa Fluor488-anti-guinea-pig	ab150185	Abcam, UK	1:200
IHC	Anti-p16 ^{INK4a}	705-4713	Roche, Germany	ready to use	HRP anti-mouse	MP7402	Vector Laboratories, USA	ready to use
IF	Anti-vimentin	61013	Progen, Germany	1:100	Cy3–anti-mouse	715165151	Jackson Immuno Research, UK	1:200

Table S3. Clinical data of patient collective. Abbreviations: Y, years; HPV, human papillomavirus; Gy, Gray; pos, positive; neg, negative; n/a, not applicable; TNM, TNM Classification of Malignant Tumors.

sample #	age by time of surgery	localisation	HPV-Status	TNM	adjuvant Radiotherapy (dose)	adjuvant Chemo-therapy	last check up (after surgery)	timing of first indication of relapse (after surgery)	details	invasion pattern (3D-OTC)
HNSCC1	72 Y	Oral Cavity	n/a	T3N1M0	yes (54 Gy)	no	32 months	17 months	local recurrence	invasive
HNSCC2	60 Y	Oral Cavity	n/a	T4aN0M0	yes (n/a)	no	20 months	n/a	no evidence of disease	expansive
HNSCC3	84 Y	Oral Cavity	n/a	T2N0M0	no	no	18 months	n/a	no evidence of disease	silent
HNSCC4	58 Y	Tonsil	pos	T2N1M0	yes (54 Gy)	yes	29 months	n/a	no evidence of disease	expansive
HNSCC5	65 Y	Tonsil	pos	T2N1M0	no	no	21 months	n/a	no evidence of disease	silent
HNSCC6	67 Y	Tonsil	neg	T2N2aM0	no	no	7 months	n/a	no evidence of disease	silent
HNSCC7	79 Y	Nasal Cavity	n/a	T4aN0M0	no	no	13 months	n/a	no evidence of disease	invasive
HNSCC8	47 Y	Tonsil	pos	T2N0M0	no	no	14 months	n/a	no evidence of disease	expansive
HNSCC9	64 Y	Tonsil	pos	T1N1M0	yes (54 Gy)	no	22 months	n/a	no evidence of disease	expansive
HNSCC10	56 Y	Hypopharynx	pos	T2N0M0	no	no	14 months	n/a	no evidence of disease	silent
HNSCC11	51 Y	Larynx	neg	T4aN1M0	yes (66 Gy)	yes	21 months	n/a	no evidence of disease	silent
HNSCC12	63 Y	Tonsil	neg	T3N0MX	n/a	no	20 months	n/a	no evidence of disease	expansive
HNSCC13	74 Y	Tonsil	pos	T2N1M0	yes (57,6 Gy)	yes	20 months	n/a	no evidence of disease	silent

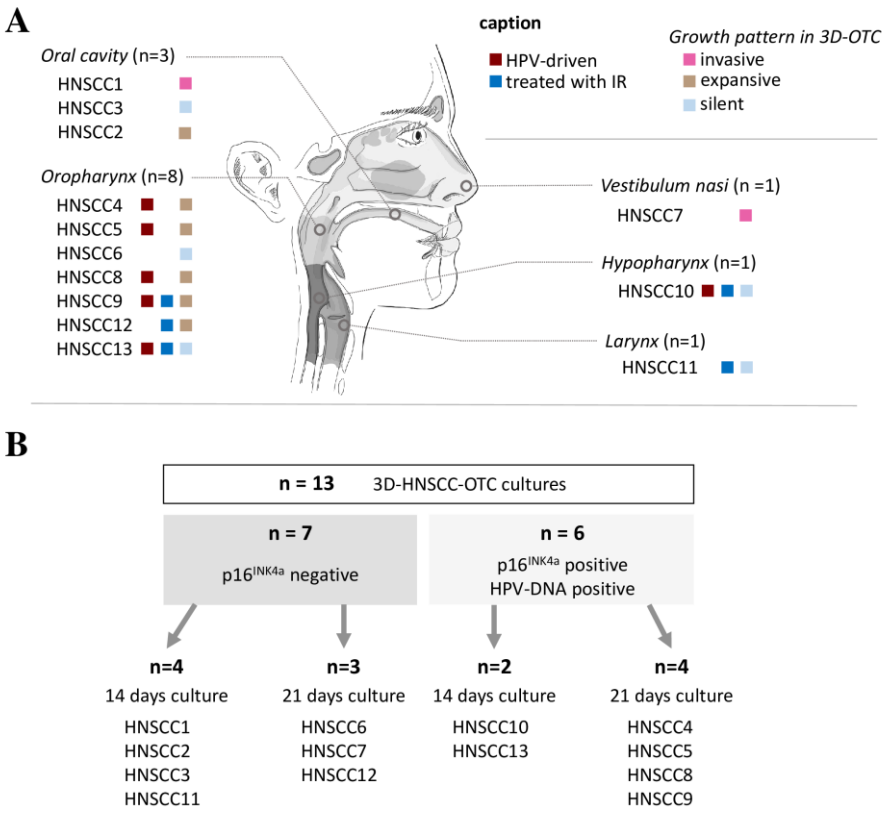

Figure S1. Specimen origin and experimental setting. **(A)** Depiction of specimen origin, HPV-status, treatment with fractionated IR and growth-pattern of 3D-OTC. **(B)** Details of HPV-status and time in culture of 3D-OTC. Abbreviations: IR, irradiation; HPV, human papillomavirus; 3D-OTC, 3D organotypic co-culture.

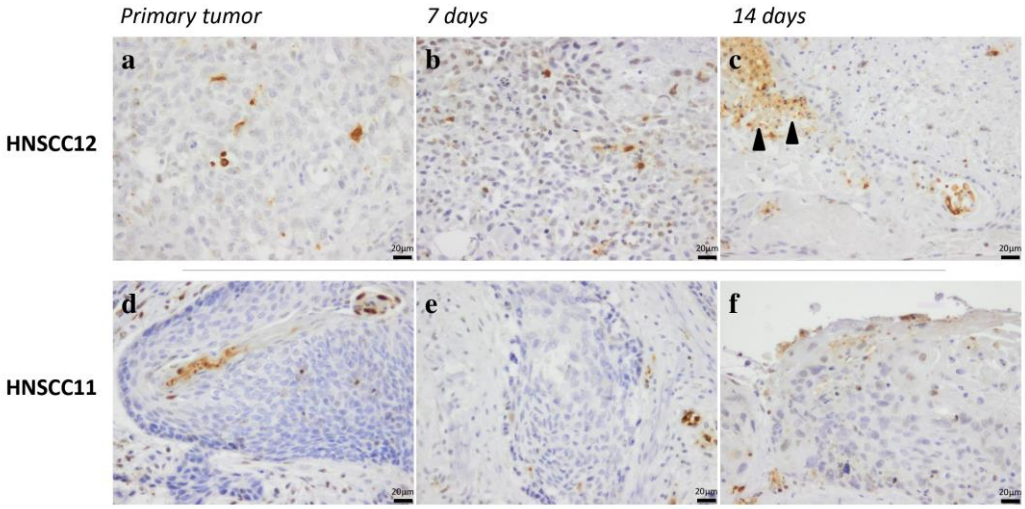

Figure S2. Visualization of apoptosis at different time points. Representative images of immunohistochemistry with an anti-cleaved caspase-3 antibody of two different HPV non-driven 3D-OTC cultures (*b-c* and *e-f*) and matching primary tumors (*a* and *d*) for visualization of apoptosis. HNSCC12 shows low levels of apoptotic cells in the primary with a slight increase during cultivation (see black arrowheads). HNSCC11 constantly low expression of cleaved caspase-3 presents in the

primary tumor and in 3D-OTC cultures. Abbreviations: HPV, human papillomavirus; 3D-OTC, 3D organotypic co-culture.

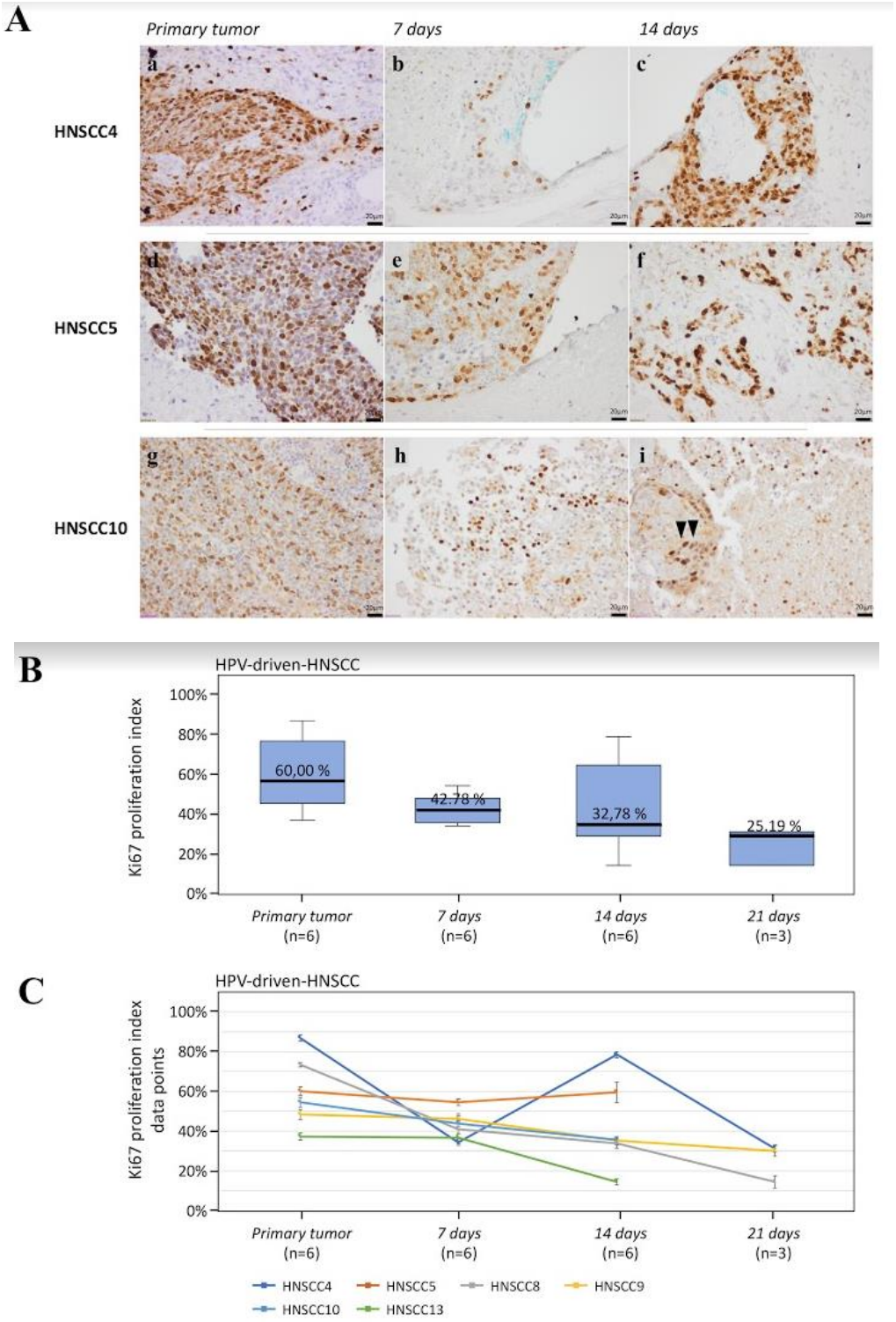

Figure S3. (A) IHC with an anti-ki-67-antibody of three different HPV-driven 3D-HNSCC-OTC (*b-c*; *e-f*; *h-i*) for the indicated time points and according primaries (*a*, *d*, *g*). **(B)** Boxplot of ki-67 proliferation indices of primaries and 3D- OTCs on day 7, 14, and 21 of all HPV driven HNSCC. **(C)** Mean values of ki-67 proliferation indices of all primaries and 3D-OTCs on day 7, 14, and 21 of all HPV driven HNSCC. Error bars indicate standard errors of the mean. Abbreviations: IHC, immunohistochemistry; HNSCC, Head and neck squamous cell carcinoma; 3D-OTC, 3D organotypic co-culture.

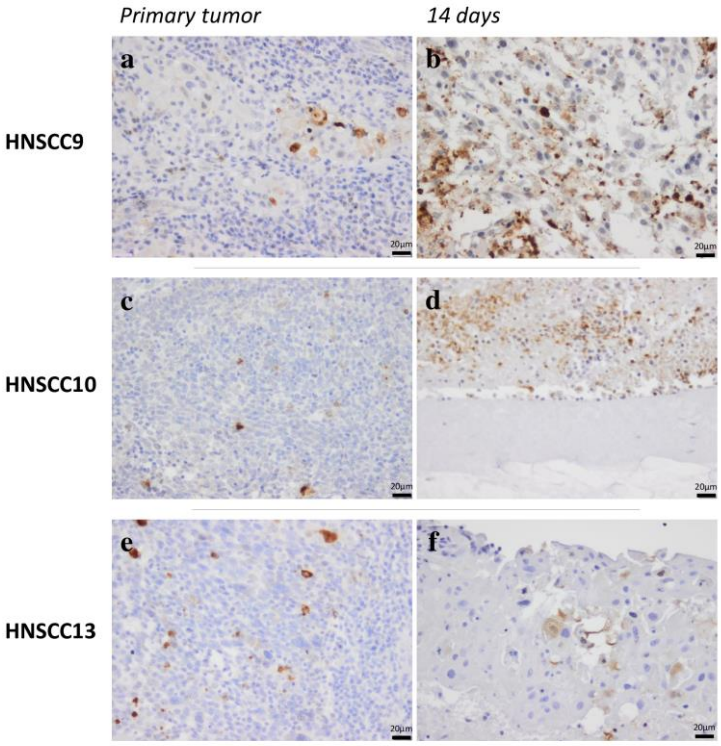

Figure S4. IHC with an anti-cc-3 antibody of three HPV-driven 3D-HNSCC-OTC on day 14 (*b, d, f*) and according primaries (*a, c, e*). HNSCC9 and HNSCC10 present an increase of cc-3 expression in 3D-HNSCC-OTC, compared to their primary. HNSCC13 maintains stable expression of cc-3 during culture. Abbreviations: IHC, immunohistochemistry; HPV, human papillomavirus; 3D-OTC, 3D organotypic co-culture; cc-3, cleaved caspase-3.

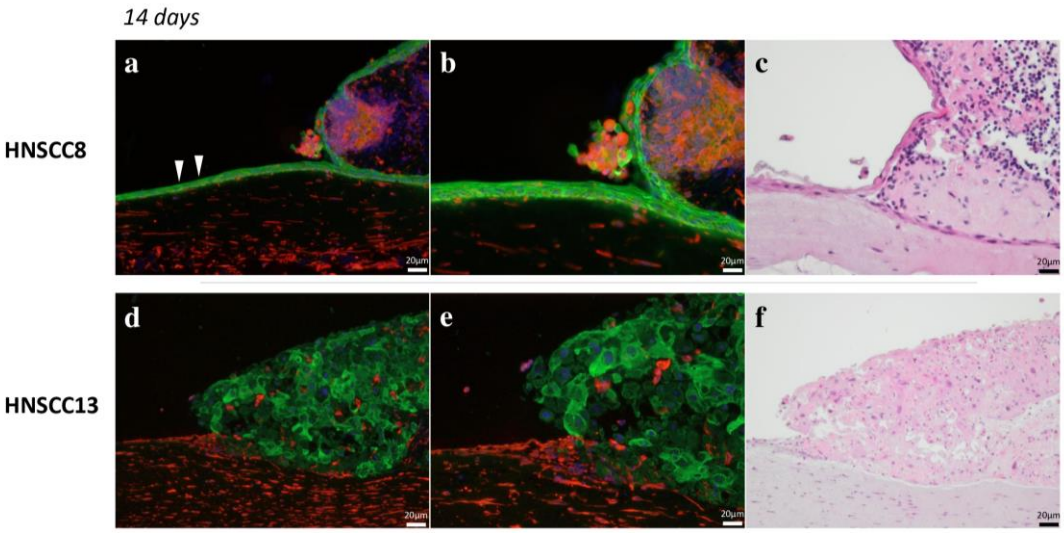

Figure S5. Co-Immunofluorescence staining with an anti-PanCK-antibody (green), an anti-vimentin-antibody (red) and DAPI (blue) (*a-b; d-e*) and H/E staining (*c, f*) of two different HPV-driven 3D-OTC on day 14. HNSCC8 presents an expansive growth pattern, white arrowheads (a) indicate the MF, HNSCC13 shows a silent growth pattern. Abbreviations: PanCK, pan-cytokeratin; H/E, Hematoxylin/eosin; HPV, human papillomavirus; 3D-OTC, 3D organotypic co-culture; MF, migration front.

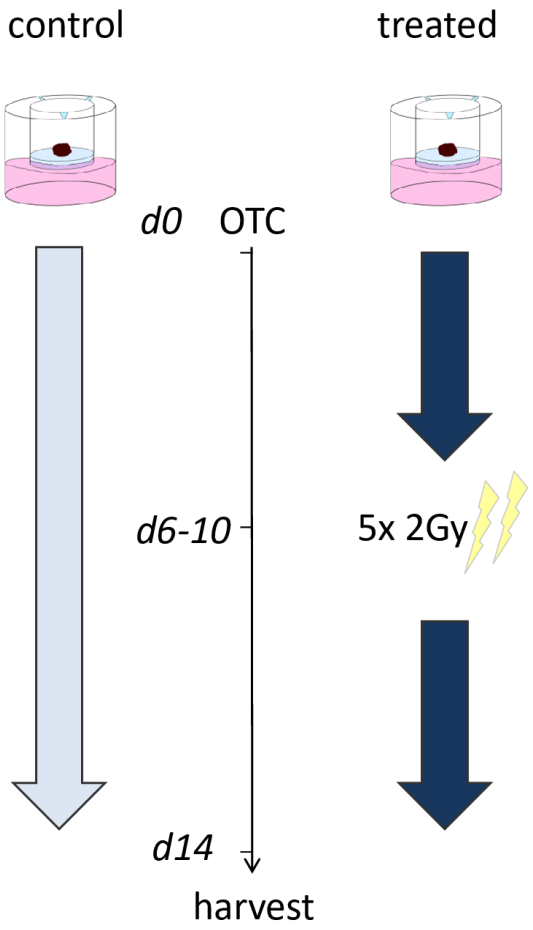

Figure 6. Diagram showing treatment with fractionated IR. After 5 days in culture 5 HNSCC-OTC were irradiated with 2 Gy on 5 consecutive days followed by 3 holidays before being harvested on day 14. Controls were mock-treated. Abbreviations: IR, irradiation; HNSCC, Head and neck squamous cell carcinoma; 3D-OTC, 3D organotypic co-culture.

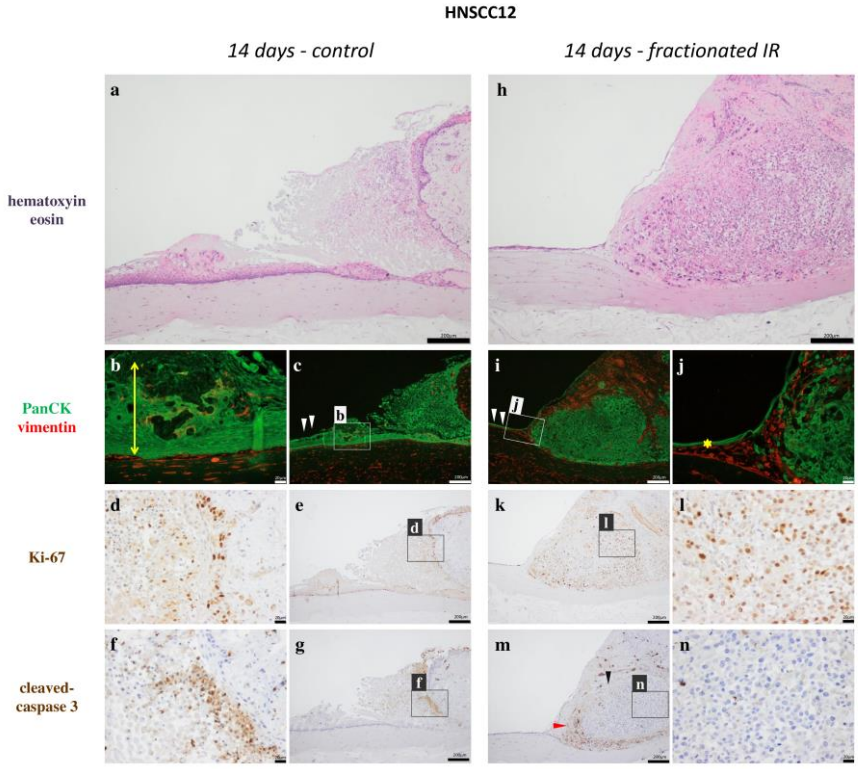

Figure 7. Selection of representative images of H/E, IF and IHC with indicated antibodies of one 3D-OTC, which was cultured for 14 days and treated with mock irradiation (*a-g*) or a fractionated irradiation scheme (*h-n*), in order to depict radiogenic impact. PanCK-vimentin-Co-IF reveals a reduction of layering in the migration front after fractionated irradiation (*see yellow flashs*) in comparision to the untreated sample. ki-67-IHC detects stable postradiogenic proliferation and IHC while cleaved caspase-3 shows heterogenous expression in the mock-treated and fractionated irradiated OTC. Apoptotic tumour cells (*see red arrowhead*) and those, not undergoing apoptosis (*see black arrowhead*). Abrreviations: H/E, haematoxylin/eosin; IF, immunofluorescence; IHC, immunohistochemistry; 3D-OTC, 3D organotypic co-culture; PanCK, pan-cytokeratin; IR, irradiation.

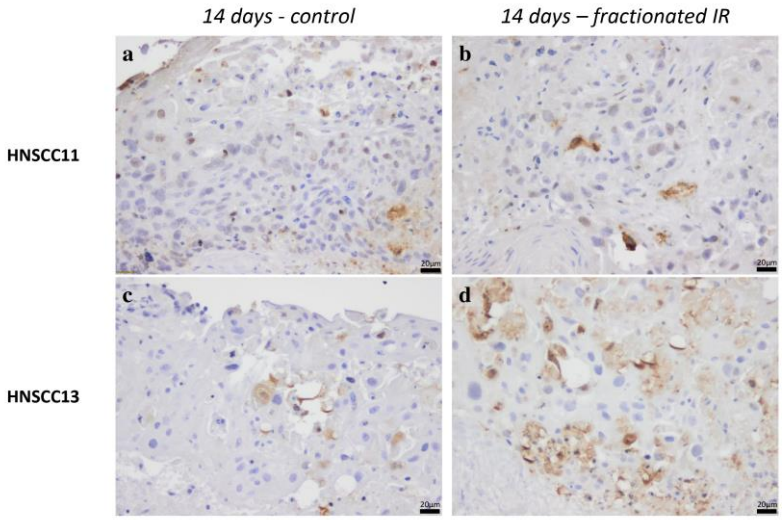

Figure S8. Impact of fractionated irradiation on apoptosis. Representative pictures of IHC with an anti-cc-3 antibody on 3D-OTC (all on day 14) of two different tumors; mock - (*a* and *c*) and matching fractionated-irradiated samples (*b* and, *d*), respectively. HNSCC11 shows similar intensity and

distribution of the cc-3 signal in the untreated OTC as well as after fractionated IR. Increasing cc-3 expression of fractional irradiated HNSCC13 compared to the mock-treated correlate. Abbreviations: IHC, immunohistochemistry; cc-3, cleaved caspase-3; 3D-OTC, 3D organotypic co-culture; IR, irradiation.

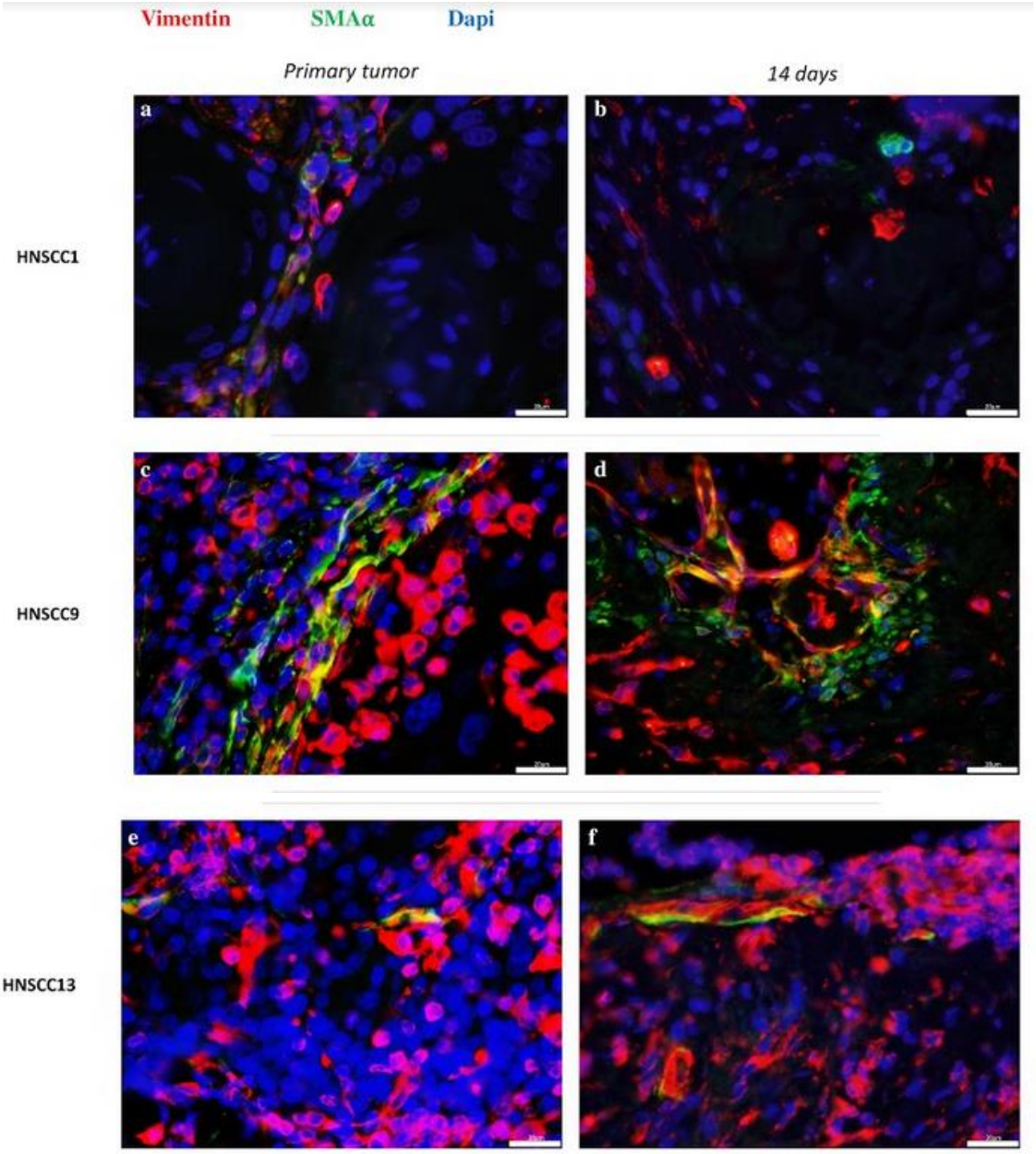

Figure S9. Representative pictures of co-IF staining for vimentin (red), ∝SMA (green) and DAPI (blue) of non-HPV-driven HNSCC1 (invasive type) and two HPV-driven HNSCC (HNSCC9; expansive type and HNSCC13; silent type in 3D-OTC on day 14 (*c, d, e*) and according primary tumor (*a, c, e*). Respective samples show a similar amount of ∝SMA positive cells in all primaries and according 3D-OTC on day 14. Abbreviations: IF, immunofluorescence; ∝SMA, ∝smooth muscle actin; HPV, human papillomavirus; HNSCC, Head and neck squamous cell carcinoma.